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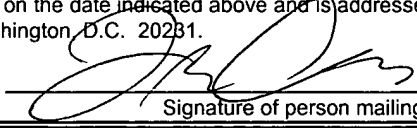
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APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

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TITLE : HUMAN METHIONINE SYNTHASE REDUCTASE:  
CLONING, AND METHODS FOR EVALUATING RISK OF  
NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE,  
CANCER, AND DOWN'S SYNDROME

5      HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND  
METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS,  
CARDIOVASCULAR DISEASE, CANCER, AND DOWN'S SYNDROME

Cross-Reference to Related Applications

This application is a Continuation-In-Part of U.S. Application Serial No. 09/371,347 entitled "Human Methionine Synthase Reductase: Cloning, and Methods for Evaluating Risk of Neural Tube Defects, Cardiovascular Disease, and Cancer" filed by Gravel et al. on August 10, 1999, which claims priority to U.S. Continuation-In-Part Application Serial No. 09/232,028 entitled "Methods for Evaluating Risk of Neural Tube Defects, Cardiovascular Disease, and Cancer" filed by Gravel et al. on January 15, 1999, which claims priority from U.S. Provisional Application No. 60/071,622, filed January 16, 1998.

15      Field of the Invention

This invention relates to the diagnosis and treatment of patients at risk for disorders associated with altered methionine synthase activity.

Background of the Invention

20      Methionine is an essential amino acid in mammals that is required for protein synthesis. Methionine also plays a central role in metabolic reactions involving transfer of single-carbon moieties: in its activated form, S-adenosylmethionine, methionine is the methyl donor in hundreds of biological transmethylation reactions. Moreover, methionine is the propylamine donor in

polyamine synthesis. The ultimate product resulting from the demethylation of methionine is homocysteine, the remethylation of which is catalyzed by a cobalamin-dependent enzyme, methionine synthase (5-methyltetrahydrofolate:homocysteine methyltransferase, EC 2.1.1.13).

5           The enzyme-bound cobalamin cofactor of methionine synthase plays an essential role in the methyl transfer reaction by acting as an intermediate methyl carrier between methyltetrahydrofolate and homocysteine. The upper portion of Fig. 1 illustrates the transfer of the methyl group of methyltetrahydrofolate ( $\text{CH}_3\text{-THF}$ ) to homocysteine via methionine synthase-methylcobalamin [MetSyn- $\text{CH}_3\text{-Co(III)}$ ] as an intermediate methyl carrier. Cleavage of the methyl-cobalt bond of the methylcob(III)alamin intermediate occurs heterolytically so as to leave the cobalamin in the highly reactive cob(I)alamin oxidation state. The occasional oxidation of the enzyme-cobalamin to the cob(II)alamin state [MetSyn-Co(II)] renders the enzyme inactive.

15           Severe deficiency of methionine synthase activity leads to megaloblastic anemia, developmental delay, hyperhomocysteinemia, and hypomethioninemia. Moreover, elevated plasma homocysteine is a risk factor in cardiovascular disease and neural tube defects (Rozen, *Clin. Invest. Med.* 19:171-178, 1996).

20           Two forms of methionine synthase deficiency are known (Watkins et al., *Am. J. Med. Genet.* 34:427-434, 1989; Gulati et al., *J. Biol. Chem.* 272:19171-19175, 1997). The first is a primary defect of the amino acid sequence of the methionine synthase enzyme. We recently cloned cDNAs encoding human methionine synthase and showed that patients from the *cblG* complementation group of folate/cobalamin metabolism have mutations in the methionine synthase gene. A second class of patients, belonging to a distinct complementation group,

*cblE*, is also deficient in methionine synthase enzymatic activity. The genetic basis of this deficiency has not been determined.

An analogous methylcobalamin-dependent methionine synthase has been well characterized in *E. coli* and the structures comprising its C-terminal half have been elucidated by X-ray crystallography. The reductive activation system required for its maintenance is a two-component flavoprotein system consisting of flavodoxin (a small FMN-containing electron transfer protein), and NADPH-ferredoxin (flavodoxin) oxidoreductase, a member of a family of electron transferases termed the "FNR family." However, flavodoxins are not found in mammalian cells.

It would be desirable to identify the enzyme that catalyzes the reductive activation of methionine synthase, i.e., the methionine synthase reductase. Knowledge of the reductase wild-type nucleotide and amino acid sequences would allow the identification of mutations and polymorphisms associated with diseases involving methionine metabolism. Moreover, an understanding of the reductase structure and function will facilitate the identification of compounds that modulate its activity. Such compounds will be useful in treating and preventing disease and developmental defects.

#### Brief Description of the Drawings

Fig. 1 is a diagram showing the enzymatic reaction that is catalyzed by methionine synthase, and the reductive reactivation of methionine synthase.

Fig. 2 is a diagram showing the overlapping clones and PCR fragments used to clone and sequence human methionine synthase reductase.

Fig. 3 is a diagram showing the nucleotide and deduced amino acid

sequence of human methionine synthase reductase.

Fig. 4 is a diagram showing an amino acid sequence comparison among human methionine synthase reductase (HsMTRR; SEQ ID NO: 21), *C. elegans* putative methionine synthase reductase (CeMTRR; SEQ ID NO: 22) and human  
5 cytochrome P450 reductase (HsCPR; SEQ ID NO: 23).

Figs. 5A and 5B are representations of Northern blots showing an analysis of methionine synthase reductase expression in human tissues.

Fig. 6 is a diagram summarizing the FISH mapping of the methionine synthase reductase gene to human chromosome 5p15.2-p15.3.

10 Figs. 7A and 7B are representations of gels showing a mutation analysis of *cblE* patient cell lines.

Fig. 7C is a diagram showing a sequence comparison of the NADPH binding region of FNR family members (SEQ ID NOs: 25-40)

15 Fig. 8A is a representation of two autoradiograms showing the A to G polymorphism at MTRR coding position 66.

Fig. 8B is a representation of a gel showing a restriction digest assay for distinguishing between the adenine 66 and guanine 66 alleles.

### Summary of the Invention

20 We have cloned the gene encoding human methionine synthase reductase. This enzyme maintains methionine synthase in its reduced, activated state, and hence is an essential component of the methionine synthetic pathway. Deficiency of methionine synthase reductase results in hyperhomocysteinemia, a condition that has been implicated in cardiovascular disease and neural tube defects. The presence of mutations in the methionine synthase reductase gene that

decrease methionine synthase reductase enzymatic activity are likely to be associated with altered risk for cardiovascular disease, neural tube defects, and cancer. The invention features methods for risk detection and treatment of patients with hyperhomocysteinemia, cardiovascular disease, neural tube defects, and  
5 cancer. The invention also features compounds and kits which may be used to practice the methods of the invention, methods and compounds for treating or preventing these conditions and methods of identifying therapeutics for the treatment or prevention of these conditions.

In a first aspect, the invention features substantially pure nucleic acid  
10 encoding a mammalian methionine synthase reductase polypeptide. In various embodiments, the nucleic acid may encode a human polypeptide, and the nucleic acid may be DNA, particularly genomic DNA or cDNA. In another embodiment, the nucleic acid has the sequence of SEQ ID NO: 1 or SEQ ID NO: 41, or degenerate variants thereof, and the nucleic acid encodes the amino acid sequence  
15 of SEQ ID NO: 2 or SEQ ID NO: 42. In yet another embodiment, the nucleic acid is operably linked to regulatory sequences for expression of methionine synthase reductase. The regulatory sequences comprise a promoter, and the promoter may be inducible.

In a second, related aspect, the invention features a substantially pure  
20 nucleic acid that hybridizes at high stringency to the nucleic acid of SEQ ID NO: 1 or SEQ ID NO: 41. In a preferred embodiment, the nucleic acid is a naturally occurring variant of the mammalian methionine synthase reductase gene. In another embodiment, the nucleic acid has a sequence complementary to at least 50% of at least 60 nucleotides of the nucleic acid encoding the methionine  
25 synthase reductase polypeptide, and the sequence is sufficient to allow nucleic acid

hybridization under high stringency conditions. In further embodiments, the nucleic acid may be a probe or an antisense nucleic acid, and the sequence may be complementary to at least 90% of at least 18 nucleotides of the nucleic acid encoding the methionine synthase reductase polypeptide.

5 In a third aspect, the invention features a nucleic acid encoding a mutant or polymorphic mammalian methionine synthase reductase polypeptide. In one embodiment, the nucleic acid may be from a human. In another embodiment, the mutation is a deletion mutation, for example, a deletion of 4 bases starting from base 1675 (bases 1675-1678) of SEQ ID NO:1 (SEQ ID NO: 47), or a deletion of  
10 3 bases starting from base 1726 (bases 1726-1728) of SEQ ID NO:1 (SEQ ID NO: 45). In still another embodiment the polymorphism is a nucleotide transition from G to A at nucleotide position 66 (SEQ ID NO: 41), or from G to A at nucleotide position 110 (SEQ ID NO: 43). Other naturally-occurring variants associated with altered risk for hyperhomocysteinemia are also a feature of this aspect of the  
15 invention.

In a fourth, related aspect, the invention features a cell containing the nucleic acid of the third aspect of the invention. In various embodiments, the cell may be a prokaryotic cell, a eukaryotic cell, a yeast cell, or a mammalian cell.

In a fifth, related aspect, the invention features a non-human transgenic  
20 animal containing the nucleic acid of the third aspect of the invention. In one embodiment, the nucleic acid contains a mutation associated with hyperhomocysteinemia.

In a sixth, related aspect, the invention features a non-human animal wherein one or both genetic alleles encoding a methionine synthase reductase  
25 polypeptide are mutated. In one embodiment of this sixth aspect, one or both

genetic alleles encoding a methionine synthase reductase polypeptide are disrupted, deleted, or otherwise rendered nonfunctional. In further embodiments of the fifth and sixth aspects, the animal may be a rodent (e.g., a mouse), or a nematode (e.g., *C. elegans*).

5                    In a seventh, related aspect, the invention features a cell from the animal of the fifth and sixth aspects.

                    In an eighth aspect, the invention features a substantially pure mammalian methionine synthase reductase polypeptide. In various embodiments, the polypeptide may be recombinant, or may be a human polypeptide, or may be  
10                   the polypeptide set forth in SEQ ID NO: 2 or SEQ ID NO: 42.

                    In a ninth, related aspect, the invention features a polypeptide having conservative amino acid substitutions relative to SEQ ID NO: 2 or SEQ ID NO: 42, and having methionine synthase reductase biological activity.

                    In a tenth, related aspect, the invention features a mutant or polymorphic  
15                   polypeptide which has less methionine synthase reductase biological activity than the polypeptide of SEQ ID NO: 2. In preferred embodiments, the polypeptide has a frameshift resulting in a premature stop codon (e.g., SEQ ID NO: 48), or a deletion mutation, such as a deletion of Leu576 (SEQ ID NO: 46). In other preferred embodiments, the polypeptide may have an amino acid substitution, such  
20                   as isoleucine instead of methionine at amino acid position 22 (SEQ ID NO: 42), or tyrosine instead of cysteine at amino acid position 37 (SEQ ID NO: 44).

                    In an eleventh, related aspect, the invention features a mutant or polymorphic polypeptide which has higher methionine synthase reductase biological activity than the polypeptide set forth in SEQ ID NO: 2.

25                   In a twelfth aspect, the invention features an antibody that specifically



binds a methionine synthase reductase polypeptide. In one embodiment, the polypeptide is a mutant or polymorphic polypeptide.

5 In a thirteenth, related aspect, the invention features a method of generating an antibody that specifically binds a methionine synthase reductase polypeptide. The method comprises administering a methionine synthase reductase polypeptide, or fragment thereof, to an animal capable of generating an immune response, and isolating the antibody from the animal. Preferred antibodies specifically bind mutant methionine synthase reductase polypeptides.

10 In a fourteenth, related aspect, the invention features a method of detecting the presence of a methionine synthase reductase polypeptide. The method comprises contacting a sample with the antibody that specifically binds a methionine synthase reductase polypeptide and assaying for binding of the antibody to the polypeptide.

15 In a fifteenth aspect, the invention features a method for detecting sequence variants for methionine synthase reductase in a mammal. The method comprises analyzing the nucleic acid of a test subject to determine whether the test subject contains a mutation or polymorphism in a methionine synthase reductase gene. The presence of the mutation or polymorphism is an indication that the animal has an increased or decreased likelihood of developing  
20 hyperhomocysteinemia, cardiovascular disease, neural tube defects, or cancer.

In one embodiment of the fifteenth aspect, primers used for detecting a mutation are selected from: 5'-CTCCTGCTCGAACATCTTCCTAAA (SEQ ID NO: 3); 5'-AATAGATAAT CCCTATCCTTATGCC (SEQ ID NO: 4); 5'-CCCTGGCTCCTAAGATATCCATC (SEQ ID NO: 5); 5'-CGAACAACAAA  
25 TTCTTTCCACTTACC (SEQ ID NO: 6); 5'-CAAGGTTGGTGGAA

GTCGCGTTG (SEQ ID NO: 7); 5'-ATGCCTTGAAGTGAT GAGGAGGTTT  
 (SEQ ID NO: 8); 5'-TTCCTACAACATAGAGAGAACTC (SEQ ID NO: 9); 5'-  
 TTGCACAAGGGCATCATGTACATC (SEQ ID NO: 10); 5'-AAACCTCC  
 TCATCACTTCAAGGCAT (SEQ ID NO: 11); 5'-CTTGCACACGAATATG  
 5 GTCTGGG (SEQ ID NO: 12); 5'-TGGCATCACCTGCATCCTTGAGG (SEQ ID  
 NO: 13); 5'-GATGTACCTGTAAATATTCTGGGGG (SEQ ID NO: 14); 5'-  
 AATCCACGGCTCAA CCACAAGTTC (SEQ ID NO: 15); 5'-CTCGAAATT  
 AACCTCACTAAAGGG (SEQ ID NO: 16); 5'-AACCCATACCGCAG  
 GTGAGCAAA (SEQ ID NO: 17); 5'-TTAGTACTTTCAGTCAAAAAA  
 10 GCTTAAT (SEQ ID NO: 18); 5'-ATAAACGACTTCAAGA GCTTGGAGC  
 (SEQ ID NO: 19); or 5'-AGGTTTGGCACTAGTAAAGCTGACT (SEQ ID NO:  
 20).

In another embodiment of the fifteenth aspect of the invention, the  
 method further comprises the step of using nucleic acid primers specific for the  
 15 methionine synthase reductase gene. The primers are used for DNA amplification  
 by the polymerase chain reaction. In yet another embodiment, the step further  
 comprises the step of sequencing nucleic acid encoding methionine synthase  
 reductase from the test subject. In still other embodiments, the analyzing includes  
 single strand conformational polymorphism (SSCP) analysis, or the method is  
 20 carried out by restriction fragment length (RFLP) polymorphism analysis. In  
 further embodiments, the method is for the diagnosis of an altered risk for  
 cardiovascular disease, neural tube defects, or cancer, such as colon cancer.

In a sixteenth aspect, the invention features a kit for the analysis of  
 mammalian methionine synthase reductase nucleic acid. The kit comprises nucleic  
 25 acid probes for analyzing the nucleic acid of a mammal, and the analyzing is

sufficient to determine whether the mammal contains a mutation in the methionine synthase reductase nucleic acid. In a preferred embodiment the nucleic acid probes allow detection of mutations associated with hyperhomocysteinemia.

In a seventeenth aspect, the invention features a kit for the analysis of  
5 mammalian methionine synthase reductase polypeptides. The kit comprises antibodies for analyzing the methionine synthase reductase polypeptide of a mammal, and the analyzing is sufficient to determine whether the mammal contains a mutation in the methionine synthase reductase nucleic acid.

In an eighteenth aspect, the invention features a method of treating or  
10 preventing cancer, cardiovascular disease, or neural tube defects. The method comprises inhibiting methionine synthase reductase biological activity. In one embodiment, the mammal is pregnant. In other embodiments, the method comprises administering a therapeutically effective dose of a methionine synthase reductase inhibitor to a mammal. The inhibitor may be a methionine synthase  
15 reductase anti-sense nucleic acid, a peptide comprising a portion of a mammalian methionine synthase reductase polypeptide, or a small molecule.

In a nineteenth aspect, the invention features a method of treating or preventing cardiovascular disease. The method comprises administering to the subject a therapeutically effective dose of a metabolite or cofactor selected from  
20 the group: folate, cobalamin, S-adenosyl methionine, betaine, or methionine.

In a twentieth aspect, the invention features a method of preventing neural tube defects, cancer, or cardiovascular disease. The method comprises: a) detecting an increased risk of neural tube defects, cancer, or cardiovascular disease, wherein the detecting is performed by analyzing methionine synthase  
25 reductase nucleic acid from one or more test subjects selected from: a mammal; a

potential parent, either male or female; a pregnant mammal; or a developing embryo or fetus, wherein the analyzing is done by the method of the fifteenth aspect of the invention; and b) exposing the mammal, potential parent, pregnant mammal, and/or developing embryo or fetus to a therapeutically effective dose of a metabolite or cofactor selected from the group: cobalamin; S-adenosyl methionine; betaine; or methionine, wherein the exposing is via the administration of the dose to the mammal, the potential parent, the pregnant mammal, and/or the developing embryo or fetus.

In a preferred embodiment of the eighteenth and twentieth aspects of the invention, the subject has been diagnosed as having a mutation or polymorphism in methionine synthase reductase.

In a twenty-first aspect, the invention features a method of screening for a compound that modulates methionine synthase reductase biological activity. The method comprises the steps of: a) contacting a sample containing wild-type, mutated, or polymorphic methionine synthase reductase with the compound, and b) assaying for methionine synthase reductase enzymatic activity, wherein increased enzymatic activity indicates an inducer of methionine synthase reductase biological activity, and decreased enzymatic activity indicates an inhibitor of methionine synthase reductase biological activity.

In a twenty-second aspect, the invention features a method for screening for a compound that modulates methionine synthase reductase biological activity. The method comprises the steps of: a) contacting a sample with the compound, and b) assaying for methionine synthase reductase expression, wherein increased expression indicates an inducer of methionine synthase reductase biological activity, and decreased expression indicates an inhibitor of methionine synthase

reductase biological activity. The sample is selected from: purified or partially purified methionine synthase reductase, a cell lysate, a cell, a nematode, or a mammal. In preferred embodiments, the sample may be the animal or cell described by the fifth and sixth aspects of the invention. In other preferred

5   embodiments, the screening may be for compounds useful for the treatment or prevention of cardiovascular disease or cancer, or for the prevention of neural tube defects.

In a twenty-third aspect, the invention features a method for detecting an increased risk of developing a neural tube defect in a mammalian embryo or fetus.

10   The method includes detecting the presence of a polymorphic methionine synthase reductase (MTRR) in a test subject, wherein the polymorphic MTRR contains a methionine instead of an isoleucine at amino acid position-22, wherein the test subject is a future parent of the embryo or fetus, and wherein detection of a homozygous MTRR polymorphism in the future parent, embryo, or fetus, or

15   detection of either a homozygous or heterozygous MTRR polymorphism in both future parents, indicates an increased risk of developing a neural tube defect in the embryo or fetus.

In various embodiments of the twenty-third aspect of the invention, the polymorphic MTRR may be detected by analyzing nucleic acid from the test

20   subject. The nucleic acid may be genomic DNA or cDNA. The nucleic acid may contain a G instead of an A at the third position of the twenty-second codon (nucleotide position 66, relative to the first nucleotide of the start codon) of MTRR.

In another embodiment of the twenty-third aspect of the invention, the

25   method may further include: a) PCR-amplifying a segment of MTRR nucleic acid

using primers MSG108S (SEQ ID NO: 49) and AD292 (SEQ ID NO: 50), and b) digesting the product of the PCR amplification reaction with the restriction enzyme *Nde* I, wherein a PCR product that is digested by *Nde* I indicates an increased risk of developing a neural tube defect in a mammalian embryo or fetus.

5           In still other embodiments of the twenty-third aspect of the invention, the polymorphic MTRR may be detected by analyzing MTRR polypeptide from the test subject, and the test subject may be a future female parent of the embryo or fetus, or the test subject may be the embryo or fetus itself.

10           In yet further embodiments of the twenty-third aspect of the invention, the method may further include detecting the presence of a polymorphic methylenetetrahydrofolate reductase (MTHFR) in a test subject, the polymorphic MTHFR having a T instead of a C at a nucleotide position equivalent to position 677 of SEQ ID NO: 51, wherein detection of the polymorphic MTHFR indicates an increased risk of developing a neural tube defect in the embryo or fetus. The  
15           polymorphic MTHFR may be detected by analyzing nucleic acid or polypeptide from the test subject.

20           In still another embodiment of the twenty-third aspect of the invention, the method may further include measuring the level of cobalamin in the test subject, wherein a low cobalamin level indicates an increased risk of developing a neural tube defect in the embryo or fetus.

25           In a twenty-fourth aspect of the present invention, the invention features a method for detecting an increased risk of developing Down's Syndrome in a mammal, preferably a mammalian embryo or fetus. In a twenty-fifth aspect the invention features a method for detecting an increased risk of developing premature coronary artery disease. Both aspects include detecting the presence of

a polymorphic MTRR in a test subject. Preferably, the polymorphic MTRR contains a common A → G polymorphism at position 66 of the MTRR cDNA sequence (SEQ ID NO:1), wherein the test subject is a mammal, preferably a future parent of an embryo or fetus or an embryo or a fetus, and wherein detection  
5 of a homozygous MTRR polymorphism indicates an increased risk of developing a Down's Syndrome or coronary artery disease defect in the embryo or fetus.

In various embodiments of the twenty-fourth aspect of the invention, the polymorphic MTRR may be detected by analyzing nucleic acid from the test subject. The nucleic acid may be genomic DNA or cDNA. The nucleic acid may  
10 contain a G instead of an A at the third position of the twenty-second codon (nucleotide position 66, relative to the first nucleotide of the start codon) of MTRR.

In another embodiment of the twenty-fourth or twenty-fifth aspects of the invention, the method may further include: a) PCR-amplifying a segment of  
15 MTRR nucleic acid using primers MSG108S (SEQ ID NO: 49) and AD292 (SEQ ID NO: 50) or A (SEQ ID NO:61) and B (SEQ ID NO:62), and b) digesting the product of the PCR amplification reaction with the restriction enzyme *Nde* I, wherein a PCR product that is digested by *Nde* I indicates an increased risk of developing a neural tube defect in a mammalian embryo or fetus.

In still other embodiments of the twenty-fourth or twenty-fifth aspects of the invention, the polymorphic MTRR may be detected by analyzing MTRR polypeptide from the test subject, and the test subject may be a future female  
20 parent of the embryo or fetus, or the test subject may be the embryo or fetus itself.

In further aspects of the invention, the invention features a method for  
25 detecting the presence of a polymorphic methylenetetrahydrofolate reductase

(MTHFR) in a test subject, (preferably an MTHFR having a T instead of a C at a nucleotide position equivalent to position 677 of SEQ ID NO: 51), wherein detection of the polymorphic MTHFR indicates an increased risk of developing Down's Syndrome in the embryo or fetus. The polymorphic MTHFR may be  
5 detected by analyzing nucleic acid or polypeptide from the test subject.

In still another embodiment of the twenty-fourth or twenty-fifth aspects of the invention, the method may further include measuring the level of cobalamin in the test subject, wherein a low cobalamin level indicates an increased risk of developing Down's Syndrome or premature cardiovascular disease in the embryo  
10 or fetus.

By "methionine synthase reductase," "methionine synthase reductase protein," or "methionine synthase reductase polypeptide" is meant a polypeptide, or fragment thereof, which has at least 43% amino acid sequence identity, or at least 53% sequence similarity, preferably at least 47% identity (or at least 57% similarity), more preferably at least 55% identity (or at least 65% similarity), yet  
15 more preferably at least 65% sequence identity (or at least 75% similarity), still more preferably at least 75% sequence identity (or at least 85% similarity) and most preferably at least 85% sequence identity (or at least 95% similarity) to the human methionine synthase reductase polypeptide of SEQ ID NO: 2 (see Fig. 4),  
20 over the length of the polypeptide or fragment thereof, or over the length of the human methionine synthase reductase polypeptide of SEQ ID NO: 2, whichever is shorter in length. It is understood that polypeptide products from splice variants of methionine synthase reductase gene sequences are also included in this definition. Preferably, the methionine synthase reductase protein is encoded by nucleic acid



having a sequence which hybridizes to a nucleic acid sequence present in SEQ ID NO: 1 (human methionine synthase reductase cDNA) under stringent conditions. Even more preferably the encoded polypeptide also has methionine synthase reductase biological activity, or is a mutant or polymorphic form of methionine synthase reductase that is associated with an increased risk of disease.

By “methionine synthase reductase nucleic acid” or “methionine synthase reductase gene” is meant a nucleic acid, such as genomic DNA, cDNA, or mRNA, that encodes methionine synthase reductase, a methionine synthase reductase protein, methionine synthase reductase polypeptide, or portion thereof, as defined above.

By “mutant methionine synthase reductase,” “methionine synthase reductase mutation(s),” “mutations in methionine synthase reductase,” “polymorphic methionine synthase reductase,” “methionine synthase reductase polymorphism(s),” “polymorphisms in methionine synthase reductase,” is meant a methionine synthase reductase (MTTR) polypeptide or nucleic acid having a sequence that confers an increased risk of a disease phenotype or enhanced protection against a disease in at least some genetic and/or environmental backgrounds. An example of a disease-associated methionine synthase reductase polymorphism is the 22M polymorphism (SEQ ID NO: 2), which is associated with an increased risk for neural tube defects.

Any given methionine synthase reductase polymorphism may be associated with an increased risk for some diseases and a decreased risk for other diseases. Increased or decreased disease risks associated with specific methionine synthase reductase mutations and polymorphisms are determined by

methods known to those skilled in the art.

Such mutations may be naturally occurring, or artificially induced.

They may be, without limitation, transition, transversion, insertion, deletion, frameshift, or missense mutations. A mutant methionine synthase reductase

5 protein may have one or more mutations, and such mutations may affect different aspects of methionine synthase reductase biological activity (protein function), to various degrees. Alternatively, a methionine synthase reductase mutation may indirectly affect methionine synthase reductase biological activity by influencing,

for example, the transcriptional activity of a gene encoding methionine synthase  
10 reductase, or the stability of methionine synthase reductase mRNA. For example, a mutant methionine synthase reductase gene may be a gene that expresses a mutant methionine synthase reductase protein or may be a gene which alters the level of methionine synthase reductase protein in a manner sufficient to confer a disease phenotype in at least some genetic and/or environmental backgrounds.

15 The presence of polymorphic or mutant methionine synthase reductase may be determined by detecting polymorphic or mutant methionine synthase reductase nucleic acid or polypeptide, using methods that are known in the art.

By “biologically active” methionine synthase reductase is meant a methionine synthase reductase protein or methionine synthase reductase gene that

20 provides at least one biological function equivalent to that of the wild-type methionine synthase reductase polypeptide or the methionine synthase reductase gene. Biological activity of a methionine synthase reductase polypeptide includes, but is not limited to, the ability to catalyze the reductive methylation of enzymatically inactive methionine synthase-cob(II)alamin to generate

enzymatically active methionine synthase-cob(III)alamin-CH<sub>3</sub>. Preferably, a biologically active methionine synthase reductase will display activity equivalent to at least 20-30% of wild-type activity, more preferably, at least 35-50% of wild-type activity, still more preferably, 55-75% of wild-type activity, and most

5 preferably, a biologically active methionine synthase reductase will display at least 80-90% of wild-type activity. A biologically active methionine synthase reductase also may display more than 100% of wild-type activity. Preferably, the biological activity of the wild-type methionine synthase reductase is determined using the methionine synthase reductase nucleic acid of SEQ ID NO: 1 or SEQ ID  
10 NO: 41 or methionine synthase reductase polypeptide of SEQ ID NO: 2 or SEQ ID NO: 42. The degree of methionine synthase reductase biological activity may be intrinsic to the methionine synthase reductase polypeptide itself, or may be modulated by increasing or decreasing the number of methionine synthase reductase polypeptide molecules present intracellularly.

15 By "high stringency conditions" is meant hybridization in 2X SSC at 40°C with a DNA probe length of at least 40 nucleotides. For other definitions of high stringency conditions, see Ausubel et al., Current Protocols in Molecular Biology, pp. 6.3.1-6.3.6, John Wiley & Sons, New York, NY, 1998, hereby incorporated by reference.

20 By "analyzing" or "analysis" is meant subjecting a methionine synthase reductase nucleic acid or methionine synthase reductase polypeptide to a test procedure that allows the determination of whether a methionine synthase reductase gene is wild-type or mutant. For example, one could analyze the methionine synthase reductase genes of an animal by amplifying genomic DNA

using the polymerase chain reaction, and then determining the DNA sequence of the amplified DNA.

By “probe” or “primer” is meant a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (the “target”). The stability of the resulting hybrid depends upon the extent of the base pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between the probe and target molecules, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art. Probes or primers specific for methionine synthase reductase nucleic acid preferably will have at least 35% sequence identity, more preferably at least 45-55% sequence identity, still more preferably at least 60-75% sequence identity, still more preferably at least 80-90% sequence identity, and most preferably 100% sequence identity. Probes may be detectably-labelled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes are used for methods involving nucleic acid hybridization, such as: nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, Northern hybridization, in situ hybridization, electrophoretic mobility shift assay (EMSA).

By “pharmaceutically acceptable carrier” means a carrier which is

physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (18<sup>th</sup> edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting, over its entire length, at least 50%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

By "identity" is meant that a polypeptide or nucleic acid sequence possesses the same amino acid or nucleotide residue at a given position, compared to a reference polypeptide or nucleic acid sequence to which the first sequence is aligned.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to

various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

5 By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a methionine synthase reductase  
10 polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure methionine synthase reductase polypeptide may be obtained, for example, by extraction from a natural source (e.g., a fibroblast) by expression of a recombinant nucleic acid encoding a methionine synthase reductase polypeptide, or by chemically synthesizing the  
15 protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system  
20 different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides not only includes those derived from eukaryotic organisms but also those synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes

which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transgene" is meant any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from that cell. Preferably the coding region of the transgene is operably linked to one or more transcriptional regulatory elements, including a promoter (as defined below) that direct transgene expression. Such a transgene may comprise a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell that includes a DNA sequence that is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic mammals (e.g., rodents such as rats or mice) and the DNA (transgene) is inserted by artifice into the genome. Transgenic organisms also may include transgenic nematodes, such as transgenic *Caenorhabditis elegans*, which are generated by methods known to those skilled in the art.

By "knockout mutation" is meant an alteration in the nucleic acid sequence that reduces the biological activity of the polypeptide normally encoded

therefrom by at least 80% relative to the unmutated gene. The mutation may, without limitation, be an insertion, deletion, frameshift mutation, or a missense mutation. Preferably, the mutation is an insertion or deletion, or is a frameshift mutation that creates a stop codon.

5 By "transformation" is meant any method for introducing foreign molecules into a cell (e.g., a bacterial, yeast, fungal, algal, plant, insect, or animal cell). Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are just a few of the methods known  
10 to those skilled in the art which may be used.

By "transformed cell" is meant a cell (or a descendant of a cell) into which a DNA molecule encoding a methionine synthase reductase polypeptide has been introduced, by means of recombinant DNA techniques.

By "positioned for expression" is meant that the DNA molecule is  
15 positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., a methionine synthase reductase polypeptide, a recombinant protein or a RNA molecule).

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which  
20 are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory



sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably at least 50%, and most preferably at least 70% amino acid sequence identity between two or more reductase family members, (e.g., between human methionine synthase reductase and human cytochrome p450 reductase). An example of a conserved region within these two reductases is the NADPH binding region (Fig. 4).

By "detectably-labeled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labeling a molecule are well known in the art and include, without limitation, radioactive labeling (e.g., with an isotope such as  $^{32}\text{P}$  or  $^{35}\text{S}$ ) and nonradioactive labeling (e.g., chemiluminescent or fluorescent labeling, e.g., fluorescein labeling).

By "antisense" as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a gene, preferably, a methionine synthase reductase gene. An antisense nucleic acid is capable of preferentially decreasing the activity of a mutant methionine synthase reductase polypeptide encoded by a mutant methionine synthase reductase gene.

By "specifically binds" is meant that an antibody recognizes and binds a human methionine synthase reductase polypeptide, but does not substantially recognize and bind other non-methionine synthase reductase molecules in a sample, e.g., a biological sample, that naturally includes protein. A preferred

antibody binds to the methionine synthase reductase polypeptide sequence of SEQ ID NO: 2 (Fig. 3).

By “neutralizing antibodies” is meant antibodies that interfere with any of the biological activities of a wild-type or mutant methionine synthase reductase polypeptide, for example, the ability of methionine synthase reductase to catalyze the transfer of a methyl group to methionine synthase-cobal(II)amin. The neutralizing antibody may reduce the ability of a methionine synthase reductase polypeptide to catalyze the transfer preferably by 10% or more, more preferably by 25% or more, still more preferably by 50% or more, yet preferably by 70% or more, and most preferably by 90% or more. Any standard assay for the biological activity of methionine synthase reductase may be used to assess potentially neutralizing antibodies that are specific for methionine synthase reductase.

By “expose” is meant to allow contact between an animal, cell, lysate or extract derived from a cell, or molecule derived from a cell, and a test compound.

By “treat” is meant to submit or subject an animal (e.g. a human), cell, lysate or extract derived from a cell, or molecule derived from a cell to a test compound.

By “test compound” is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to modulate an alteration in reporter gene activity or protein levels, by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By “assaying” is meant analyzing the effect of a treatment, be it

chemical or physical, administered to whole animals, cells, or lysates, extracts, or molecules derived therefrom. The material being analyzed may be an animal, a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be for the purpose of detecting altered protein biological activity, altered protein stability, altered protein levels, altered gene expression, or altered RNA stability. The means for analyzing may include, for example, the detection of the product of an enzymatic reaction, (e.g., the formation of active methionine synthase or methionine as a result of methionine synthase reductase activity), antibody labeling, immunoprecipitation, and methods known to those skilled in the art for detecting nucleic acids.

By “modulating” is meant changing, either by decrease or increase, in biological activity.

By “a decrease” is meant a lowering in the level of biological activity, as measured by inhibition of: a) the formation of enzymatically active methionine synthase-cob(III)alamin-CH<sub>3</sub> or methionine as a result of methionine synthase reductase activity; b) protein, as measured by ELISA; c) reporter gene activity, as measured by reporter gene assay, for example, lacZ/ $\beta$ -galactosidase, green fluorescent protein, luciferase, etc.; or d) mRNA, as measured by PCR relative to an internal control, for example, a “housekeeping” gene product such as  $\beta$ -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In all cases, the decrease is preferably by at least 10% more preferably by at least 25%, still more preferably by at least 50%, and even more preferably by at least 70%.

By “an increase” is meant a rise in the level of biological activity, as measured by a stimulation of: a) the formation of methionine synthase-

cob(III)alamin-CH<sub>3</sub> or methionine as a result of methionine synthase reductase activity; b) protein, as measured by ELISA; c) reporter gene activity, as measured by reporter gene assay, for example, lacZ/ $\beta$ -galactosidase, green fluorescent protein, luciferase, etc.; or d) mRNA, as measured by PCR relative to an internal control, for example, a “housekeeping” gene product such as  $\beta$ -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Preferably, the increase is by at least 10%, more preferably by at least 25%, still more preferably by at least 75%, even more preferably by 2-fold, and most preferably by at least 3-fold.

By “alteration in the level of gene expression” is meant a change in gene activity such that the amount of a product of the gene, i.e., mRNA or polypeptide, is increased or decreased, or that the stability of the mRNA or the polypeptide is increased or decreased.

By “reporter gene” is meant any gene that encodes a product whose expression is detectable and/or quantifiable by immunological, chemical, biochemical or biological assays. A reporter gene product may, for example, have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ/ $\beta$ -galactosidase, luciferase, chloramphenicol acetyltransferase), toxicity (e.g., ricin A), or an ability to be specifically bound by a second molecule (e.g., biotin or a detectably-labelled antibody). It is understood that any engineered variants of reporter genes, which are readily available to one skilled in the art, are also included, without restriction, in the forgoing definition.

By “protein” or “polypeptide” or “polypeptide fragment” is meant any chain of more than two amino acids, regardless of post-translational modification

(e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

5 By “missense mutation” is meant the substitution of one purine or pyrimidine base (i.e. A, T, G, or C) by another within a nucleic acid sequence, such that the resulting new codon may encode an amino acid distinct from the amino acid originally encoded by the reference (e.g. wild-type) codon.

10 By “deletion mutation” is meant the deletion of at least one nucleotide within a polynucleotide coding sequence. A deletion mutation alters the reading frame of a coding region unless the deletion consists of one or more contiguous 3-nucleotide stretches (i.e. “codons”). Deletion of a codon from a nucleotide coding region results in the deletion of an amino acid from the resulting polypeptide.

15 By “frameshift mutation” is meant the insertion or deletion of at least one nucleotide within a polynucleotide coding sequence. A frameshift mutation alters the codon reading frame at and/or downstream from the mutation site. Such a mutation results either in the substitution of the encoded wild-type amino acid sequence by a novel amino acid sequence, or a premature termination of the encoded polypeptide due to the creation of a stop codon, or both.

20 By “low serum cobalamin level” is meant a serum cobalamin concentration of less than 328 pmol/L in a child, fetus, or embryo that has a neural tube defect or is at risk for developing a neural tube defect, or a serum cobalamin concentration of less than 259 pmol/L in the mother or future parent of a child having a neural tube defect.

By “polymorphic methylenetetrahydrofolate reductase” or “mutant

methylenetetrahydrofolate reductase” is meant methylenetetrahydrofolate reductase (MTHFR) polypeptide or nucleic acid having a sequence that confers an increased risk of a disease phenotype in at least some genetic and/or environmental backgrounds, for example, in combination with an MMTR polymorphism or mutation.

By “677C→T polymorphism in MTHFR” is meant a substitution of cytosine in place of thymine in nucleic acid encoding MTHFR at a nucleotide position equivalent to MTHFR nucleotide position 677 as disclosed in Frosst et al. (*Nat. Genet.* 10:111-113, 1995) and in Genbank Accession No. U09806 (SEQ ID NO: 51).

By “future parent” is meant a male or female who has contributed or may potentially contribute genetic material (e.g., a sperm or an egg) to form a zygote. A future parent is also a female who gestates or may potentially gestate an embryo or fetus in her uterus, irrespective of whether she has contributed or may potentially contribute genetic material to the embryo or fetus; an example of such a future parent is a surrogate mother).

By “test subject” is meant a future parent as defined above, an embryo, or a fetus.

By “sample from a test subject” is meant a specimen, for example, and not limited to, blood, serum, cells, or amniotic fluid, that would allow one of skill in the art to determine whether the test subject has a mutant or polymorphic methionine synthase reductase.

By “cardiovascular disease” is meant cardiovascular disease associated with elevated plasma homocysteine as described in (Rozen, *Clin. Invest. Med.*

19:171-178, 1996). As used herein, the term cardiovascular disease includes premature coronary artery disease.

### Detailed Description of the Invention

5           Methionine synthase catalyzes the remethylation of homocysteine to methionine in a reaction in which methylcobalamin serves as an intermediate methyl carrier.

Over time, the cob(I)alamin cofactor of methionine synthase may become oxidized to cob(II)alamin, thus rendering the enzyme inactive.

10          Regeneration of the functional enzyme occurs through the reductive methylation of the cob(II)alamin in a reaction in which S-adenosylmethionine is utilized as methyl donor (Fig. 1). The reductive activation system in the lower part of the scheme shown in Fig. 1 is the mechanism by which S-adenosylmethionine (Ado-Met) together with an electron reactivates the enzyme to the functional,  
15          methionine synthase-CH<sub>3</sub>-Co(III) state, resulting in the formation of S-adenosylhomocysteine (Ado-Hcy) as a reaction by-product.

Patients of the *cblE* complementation group of disorders of folate/cobalamin metabolism, who are defective in the reductive activation of methionine synthase, have megaloblastic anemia, developmental delay,  
20          hyperhomocysteinemia, and hypomethioninemia. We have cloned a cDNA corresponding to the "methionine synthase reductase" reducing system required for maintenance of the methionine synthase in a functional state. Using primers comprising sequences of consensus binding sites for FAD, FMN and NADPH, we performed RT-PCR and inverse PCR to clone a methionine synthase reductase

cDNA. The cDNA hybridizes to an mRNA of 3.6 kb (as detected by Northern blot). The deduced protein is a novel member of the FNR family of electron transferases, containing 698 amino acids with a predicted Mr of 77,700. It shares 38% identity with human cytochrome P450 reductase and 43% with the *C. elegans* putative methionine synthase reductase (see below). Methionine synthase reductase was localized to human chromosome 5p15.2-15.3 by fluorescence *in situ* hybridization (FISH).

A survey of the NCBI databases for homology to the human methionine synthase reductase using BLASTP or TBLASTN yielded the putative methionine synthase reductase of *C. elegans* (P value =  $9 \times 10^{-92}$ ). Proteins of the FNR family were also found using the BLAST programs. The strongest homology was found with cytochrome P450 reductase (P values  $> 3 \times 10^{-68}$ ), followed by nitric oxide synthase (three isoforms, P values  $> 4 \times 10^{-52}$ ), and sulfite reductase (P values  $> 6 \times 10^{-39}$ ). Lower, but still significant homology was found with *E. coli* NADPH-ferredoxin(flavodoxin) reductase (P values  $> 2 \times 10^{-9}$ ) and flavodoxin (P values  $> 3 \times 10^{-2}$ ). Our finding suggests a convergent evolution of the two-gene flavodoxin/NADPH-ferredoxin(flavodoxin) reductase system to a single gene encoding a fused version of the two proteins in human cells. Alignment of the proteins provides for a large linker region bridging the two components.

The identity of our cloned cDNA sequence as that encoding methionine synthase reductase was confirmed by the identification of mutations in the corresponding gene in *cblE* patients having a functional deficiency of methionine synthase. Our key finding confirming the identification of the cDNA was a 4 bp frameshift mutation in two affected siblings. The occurrence of a functionally null



mutation in a candidate gene provides compelling evidence that the mutation is causative of disease in the affected patients. Furthermore, a 3 bp deletion detected in a third patient is also highly likely to cause an enzyme defect, and the direct sequencing of PCR products suggested that the patient's second allele contains a  
5 mutation that renders the mRNA very unstable or poorly transcribed. In all, seven of ten tested *cblE* cell lines showed evidence of mutation although the sequence changes have yet to be determined in the remaining four.

The two mutations we have identified associated with *cblE* disease are located in the vicinity of the NADPH binding domain by comparison with proteins  
10 of the FNR family. The 4 bp deletion yields a truncated protein that is expected to be deficient in NADPH binding and possibly in FAD binding, since the C-terminus of the enzyme may be involved in both. The 3 bp deletion results in the deletion of Leu576, which is located between two sequences that may be involved in NADPH binding. Leu576 is well conserved among reductases that are  
15 similar to the methionine synthase reductase (Fig. 6C). This supports the idea that deletion of the Leu576 codon (1726delTTG) results in an enzymatic defect, although confirmation will require expression of the mutant protein. This residue is also conserved in the NADPH-ferredoxin (flavodoxin) reductase enzymes of several organisms, although the homology with this portion of the protein is low or  
20 absent in some cases. It is possible that the deletion affects the relationship between the two NADPH-binding sequences that are in its vicinity.

The cloning of human methionine synthase reductase cDNA enables the determination of the enzymatic mechanism involved in the reductive activation of methionine synthase. Furthermore, it is now possible to identify additional

mutations in patients with severe deficiency of the enzyme activity, and to determine whether there exist common amino acid polymorphisms which lead to mildly elevated homocysteine levels. Such elevations may be a risk factor in cardiovascular disease, neural tube defects, and cancer.

- 5           Mutations in the human methionine synthase reductase gene that result in altered homocysteine and/or folate levels may be risk factors for the diseases listed above. The methods of the invention therefore provide diagnostic assays for such risk factors, as well as methods of treating or preventing cardiovascular disease, neural defects, cancer, megaloblastic anemia, and hypomethioninemia. In addition, the invention provides methods for screening assays for the isolation of potential therapeutic compounds that modulate methionine synthase reductase activity.

- 15           The assays described herein can be used to test for compounds that modulate methionine synthase activity and hence may have therapeutic value in the prevention of neural tube defects, prevention and/or treatment of cancer, cardiovascular disease, homocysteinemia, and megaloblastic anemia.

### Test Compounds

- 20           In general, novel drugs for prevention of neural tube defects, or prevention and/or treatment of cancer, cardiovascular disease, and megaloblastic anemia are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening

procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their therapeutic activities for homocysteinemia, megaloblastic anemia, cardiovascular disease, cancer, and neural tube defects should be employed whenever possible.

When a crude extract is found to modulate methionine synthase reductase biological activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract that modulates methionine synthase reductase biological activity. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using mammalian models of homocysteinemia, megaloblastic anemia, cardiovascular disease, cancer, and neural tube defects.

Methionine synthase reductase assays for the detection of compounds that modulate methionine synthase reductase activity and expression

Potentially useful therapeutic compounds that modulate (e.g. increase or decrease) methionine synthase reductase activity or expression may be isolated by various screens that are well-known to those skilled in the art. Such compounds may modulate methionine synthase reductase expression at the pre- or post-transcriptional level, or at the pre- or post-translational level.

A. Screens for compounds that modulate methionine synthase reductase enzymatic

## activity

Screens for potentially useful therapeutic compounds that modulate methionine synthase reductase activity may be readily performed. For example, the effect of a test compound on methionine synthase reductase activity may be determined by measuring formation of  $^{14}\text{CH}_3\text{-cob(III)alamin}$ , which results from the transfer of  $^{14}\text{CH}_3$  from S-adenosylmethionine to methionine synthase-cob(II)alamin. A test compound that increases the enzymatic activity of a methionine synthase reductase would result in increased levels of methionine synthase- $^{14}\text{CH}_3\text{-cob(III)alamin}$ , and a compound that decreases the enzymatic activity of a methionine synthase reductase would result in decreased levels of methionine synthase- $^{14}\text{CH}_3\text{-cob(III)alamin}$ .

The effect of a test compound on methionine synthase reductase activity also may be determined by measuring the resulting activity of methionine synthase. The amount of reaction product (i.e., methionine) formation reflects the relative activity of methionine synthase, which in turn reflects the relative activity of methionine synthase reductase, which in turn indicates the effect of the test compound on methionine synthase reductase activity. For example, a sample containing methionine synthase and homocysteine may contain a mutant, inactive methionine synthase reductase which does not reduce oxidized methionine synthase, and hence, no methionine is formed. However, a test compound that increases the enzymatic activity of the mutant methionine synthase reductase will result in increased levels of methionine formation, relative to control samples not containing the test compound. Analogously, a compound that decreases methionine synthase reductase activity will result in the formation of decreased

levels of methionine formation in reactions containing active methionine synthase reductase. That a test compound directly modulates methionine synthase reductase enzymatic activity, as opposed to methionine synthase enzymatic activity, can be confirmed by including control reactions that lack methionine synthase reductase.

- 5 Such control reactions should not show altered levels of methionine production if the test compound directly modulates methionine synthase reductase activity.

Examples of methionine synthase activity assays, *in vitro* and in whole cells, are well-known to those skilled in the art (see, for example, Gulati et al., 1997, *J. Biol. Chem.* 272:19171-19175; see also Rosenblatt et al., 1984, *J. Clin.*

- 10 *Invest.* 74:2149-2156).

#### B. ELISA for the detection of compounds that modulate methionine synthase reductase expression

Enzyme-linked immunosorbant assays (ELISAs) are easily incorporated into high-throughput screens designed to test large numbers of compounds for their ability to modulate levels of a given protein. When used in the methods of the invention, changes in a given protein level of a sample, relative to a control, reflect changes in the methionine synthase reductase expression status of the cells within the sample. Protocols for ELISA may be found, for example, in Ausubel et

- 15 al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997. Lysates from cells treated with potential modulators of methionine synthase reductase expression are prepared (see, for example, Ausubel et al., *supra*), and are loaded onto the wells of microtiter plates coated with “capture” antibodies specific for methionine synthase reductase. Unbound antigen is washed out, and a
- 20

methionine synthase reductase-specific antibody, coupled to an agent to allow for detection, is added. Agents allowing detection include alkaline phosphatase (which can be detected following addition of colorimetric substrates such as *p*-nitrophenolphosphate), horseradish peroxidase (which can be detected by chemiluminescent substrates such as ECL, commercially available from Amersham) or fluorescent compounds, such as FITC (which can be detected by fluorescence polarization or time-resolved fluorescence). The amount of antibody binding, and hence the level of a methionine synthase reductase polypeptide within a lysate sample, is easily quantitated on a microtiter plate reader.

- 10 As a baseline control for methionine synthase reductase expression, a sample that is not exposed to test compound is included. Housekeeping proteins are used as internal standards for absolute protein levels. A positive assay result, for example, identification of a compound that increases or decreases methionine synthase reductase expression, is indicated by an increase or decrease in
- 15 methionine synthase reductase polypeptide within a sample, relative to the methionine synthase reductase level observed in cells which are not treated with a test compound.

### C. Reporter gene assays for compounds that modulate methionine synthase reductase expression

- 20 Assays employing the detection of reporter gene products are extremely sensitive and readily amenable to automation, hence making them ideal for the design of high-throughput screens. Assays for reporter genes may employ, for example, colorimetric, chemiluminescent, or fluorometric detection of reporter

gene products. Many varieties of plasmid and viral vectors containing reporter gene cassettes are easily obtained. Such vectors contain cassettes encoding reporter genes such as lacZ/ $\beta$ -galactosidase, green fluorescent protein, and luciferase, among others. Cloned DNA fragments encoding transcriptional control regions of interest (e.g. that of the mammalian methionine synthase reductase gene) are easily inserted, by DNA subcloning, into such reporter vectors, thereby placing a vector-encoded reporter gene under the transcriptional control of any gene promoter of interest. The transcriptional activity of a promoter operatively linked to a reporter gene can then be directly observed and quantitated as a function of reporter gene activity in a reporter gene assay.

Cells are transiently- or stably-transfected with methionine synthase reductase control region/reporter gene constructs by methods that are well known to those skilled in the art. Transgenic mice containing methionine synthase reductase control region/reporter gene constructs are used for late-stage screens *in vivo*. Cells containing methionine synthase reductase/reporter gene constructs are exposed to compounds to be tested for their potential ability to modulate methionine synthase reductase expression. At appropriate timepoints, cells are lysed and subjected to the appropriate reporter assays, for example, a colorimetric or chemiluminescent enzymatic assay for lacZ/ $\beta$ -galactosidase activity, or fluorescent detection of GFP. Changes in reporter gene activity of samples treated with test compounds, relative to reporter gene activity of appropriate control samples, indicate the presence of a compound that modulates methionine synthase reductase expression.



D. Quantitative PCR of methionine synthase reductase mRNA as an assay for compounds that modulate methionine synthase reductase expression

The polymerase chain reaction (PCR), when coupled to a preceding reverse transcription step (rtPCR), is a commonly used method for detecting vanishingly small quantities of a target mRNA. When performed within the linear range, with an appropriate internal control target (employing, for example, a housekeeping gene such as actin), such quantitative PCR provides an extremely precise and sensitive means of detecting slight modulations in mRNA levels. Moreover, this assay is easily performed in a 96-well format, and hence is easily incorporated into a high-throughput screening assay. Cells are treated with test compounds for the appropriate time course, lysed, the mRNA is reverse-transcribed, and the PCR is performed according to commonly used methods, (such as those described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997), using oligonucleotide primers that specifically hybridize with methionine synthase reductase nucleic acid. Changes in product levels of samples exposed to test compounds, relative to control samples, indicate test compounds that modulate methionine synthase reductase expression.

Secondary screens of test compounds that appear to modulate methionine synthase reductase activity

After test compounds that appear to have methionine synthase reductase-modulating activity are identified, it may be necessary or desirable to subject these compounds to further testing. At late stages testing will be

performed *in vivo* to confirm that the compounds initially identified to affect methionine synthase reductase activity will have the predicted effect *in vivo*. Such tests may be performed using cells or animals that have wild-type, mutated, or deleted methionine synthase reductase genes, or wild-type or mutated methionine synthase reductase transgenes.

### Therapy

Compounds identified using any of the methods disclosed herein, may be administered to patients or experimental animals with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients or experimental animals. Although intravenous administration is preferred, any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or

hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for antagonists or agonists of the invention include  
5 ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

10 The following examples are to illustrate, not limit the invention.

#### EXAMPLE 1: GENERAL METHODS

##### Materials

Radiolabeled compounds were from DuPont (Wilmington, DE). A human multiple tissue Northern blot and  $\beta$ -actin probe were from Clontech (Palo Alto, CA). The random-primed DNA labelling kit was from Boehringer  
15 Mannheim (Indianapolis, IN). The T/A cloning kit was from Invitrogen (Carlsbad, CA), the Geneclean III kit was obtained from Bio101 Inc. (Vista, CA), and the Wizard Mini-Preps were from Promega (Madison, WI). Taq polymerase, AMV reverse transcriptase, Trizol reagent, and were purchased from Gibco BRL  
20 (Gaithersburg, MD), and restriction enzymes were purchased from GibCo BRL and New England Biolabs (Beverly, MA). The Sequenase kits for manual sequencing of crude PCR products or plasmids were from United States

Biochemicals (Cleveland, OH). The oligonucleotides (SEQ ID NOs: 3-20 and 49-50) were synthesized by ACGT Corporation (Toronto, Canada) or by the Sheldon Biotechnology Centre, McGill University. The sequences of oligonucleotides are shown in Table 1 and in Fig. 2. A human cDNA library, made in Lambda-ZAP  
5 from RNA derived from the human colon carcinoma line Caco-2, was used as template in some PCR reactions to obtain 5' extensions of the cDNA.

### Homology matches

Comparisons were made between putative FMN, FAD and NADPH binding sites and sequences in the NCBI databases (dbEST and nr) using the  
10 BLAST programs (Altschul et al., *Nat. Genet.* 6:119-129, 1994). The cytochrome P450 reductase and nitric oxide synthase full sequences were also used for homology searching.

### PCR cloning and DNA sequencing

Total cellular RNA was isolated by the method of Chirgwin et al.  
15 (*Biochemistry*, 18:5294-5299, 1979) and reverse-transcribed using oligo-dT15 as primer. PCR was conducted as described previously (Triggs et al., *Am. J. Hum. Genet.* 49:1041-1054, 1991). The PCR products were purified using GeneClean, subcloned in the pCR2.1 vector and transformed into *E. coli* according to the supplier's protocol (TA cloning kit). The resulting clones were sequenced  
20 manually to confirm the specificity of PCR products. Automated sequencing was done by Bio S&T Inc. (Montreal, Canada) or by the DNA Sequencing Core Facility of the Canadian Genetic Diseases Network.

### Northern blot

The multiple tissue Northern blot, prepared from poly(A)+ RNA (2  $\mu$ g/lane) of the indicated human tissues, was probed with an EcoRI segment of a subclone in pCRII containing an insert spanning positions 335-2148 of the methionine synthase reductase cDNA. Hybridization with human  $\beta$ -actin cDNA served as a control for the quantity and integrity of the RNA in the blot.

### Chromosomal localization

We performed PCR analysis of DNA from the NIGMS human/rodent somatic cells hybrid mapping panel (#2). The oligonucleotide primers, which were specific for the 3'-UTR region of the gene, amplified a 111 nucleotide product (accession #G19837 in dbSTS). A P1-derived artificial chromosome (PAC) clone (104K2) was identified from a total human genomic library (Ioannou, P.A. et al., *Nat. Genet.* 6:84-89, 1994) by hybridization screening with a methionine synthase reductase cDNA probe (clone 704947, accession #AA279726 in dbEST) and this genomic clone was then used for FISH mapping (Heng, H. H. et al., *Proc. Natl. Acad. Sci. USA* 89:9509-9513, 1992; Heng, H.H and Tsui, L.C., *Chromosoma* 102:325-332, 1993).

### Cell lines

Ten fibroblast cell lines from patients with homocystinuria (*cbIE* complementation group) were used to identify mutations and polymorphisms in the MTRR gene using reverse transcription-PCR of total cellular RNA. Three of the cell lines displayed mutations: WG788 from the original *cbIE* patient (Schuh et

al., *N. Engl. J. Med.* 310:686-690, 1984); WG1146 from his younger brother, who had been diagnosed before birth, and whose mother was treated with hydroxocobalamin during pregnancy (Rosenblatt et al., *Lancet* 1:1127-1129, 1985); and WG1836 from a patient who had previously been described as having dihydrofolate reductase deficiency (case 1 in Tauro et al., *N. Engl. J. Med.* 294:466, 1976) and subsequently as having a "new mutation" associated with low methylcobalamin levels and reduced cellular folate uptake (Brasch et al, *Aust. N. Z. J. Med.* 18 Supp.434, 1988). In our laboratory, we have shown that the fibroblast line from this last patient falls into the *cblE* complementation group.

The fibroblast cell line WG1401 was the first to show the polymorphism, an A to G substitution at bp 66. WG1401 is from patient B.S.S. 17, with megaloblastic anemia, hyperhomocysteinemia, and mild methylmalonic aciduria. The polymorphism was also found in a control cell line, MCH64.

Twenty-two other cell lines were used as normal controls for mutation analysis.

#### Mutation analysis by RT-PCR of fibroblast RNA

Total cellular RNA was isolated from fibroblast pellets (Chirgwin et al., *Biochemistry*, 18:5294-5299, 1979). It was reverse transcribed using 25  $\mu$ g total RNA in reactions containing 2.5 U of AMV reverse transcriptase and 500 ng of methionine synthase reductase-specific terminal oligonucleotide 2101C (SEQ ID NO: 20; Table 1) in a total reaction volume of 54  $\mu$ l. The resultant cDNA was used as template for PCR. PCR for nine overlapping cDNA segments was performed in reactions containing 3  $\mu$ l of template, 1  $\mu$ l each of dTTP, dGTP,

dATP and dCTP (10 mM), and 3 U Taq polymerase in a 46  $\mu$ l volume. PCR products were verified by agarose gel electrophoresis before testing for heteroduplex formation. Heteroduplex analysis was carried out by mixing mutant and control PCR products 1:1, heating the mixture to 95°C for 3 min, cooling to room temperature, and subjecting the samples to electrophoresis on an 8% polyacrylamide gel. Fragments displaying shifts were subcloned and sequenced, or sequenced directly.

#### MMTR polymorphism analysis in genomic DNA samples

For the screening of genomic DNA samples, restriction digestion analysis was performed with an artificially-created NdeI restriction site using the sense primer MSG108S 5'GCAAAGGCCATCGCAGAAGACAT (SEQ ID NO: 49) and antisense primer AD292 5'GTGAAGATCTGCAGAAAATCCATGTA (SEQ ID NO: 50), where the underlined C replaces the A to generate an NdeI restriction site in the normal sequence. To test for the mutation, 10  $\mu$ l of PCR product was digested by adding 6  $\mu$ l H<sub>2</sub>O, 2  $\mu$ l New England Biolab's (NEB) buffer 4 and 2  $\mu$ l NdeI. The PCR fragment of 66 bp remains uncut in the presence of the G (methionine) allele, but is digested into fragments of 44 bp and 22 bp in the presence of the A (isoleucine) allele.

#### Subjects

Patients with spina bifida (n=56) and mothers of children with spina bifida (n=58) were recruited from the Montreal Children's Hospital after approval of the protocol by the Institutional Review Board. The controls (n=97) were other

outpatients who were having a venipuncture at the Pediatric Test Center, Montreal Children's Hospital, and who were with their mothers (n=89). Blood samples were obtained from mothers and children after appropriate consent. Exclusion criteria were syndromic neural tube disorder (NTD) cases, severe anemia, neoplastic disease, renal insufficiency and immunosuppressive therapy. Individuals who were taking vitamin supplements were also excluded. The methylenetetrahydrofolate reductase (MTHFR) genotypes and the levels of plasma homocysteine and serum cobalamin were previously determined in these subjects. The concentration of serum cobalamin was quantitated by routine methods, using an automated system and reagents from Ciba (Ciba Corning Diagnostics Corp., Medfield, MA).

To determine total homocysteine (tHcy) levels in plasma, blood samples were drawn to Becton-Dickinson vacutainers containing sodium EDTA and kept on ice until plasma was separated. Plasma was separated by centrifugation for 5 min., removed, and centrifuged again; the supernatant was collected and frozen at -20°C until analysis. tHcy in plasma was determined by high pressure liquid chromatography as reported (Gilfix et al., *Clin. Chem.* 43:687-688, 1997). The tHcy adduct was detected by fluorescence after precolumn derivitization with the thiol-specific reagent 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F)(Wako, USA).

To detect the MTHFR polymorphism, DNA was isolated from peripheral leukocytes by extraction with phenol-chloroform after cell lysis in a buffer containing Nonidet-P40 (Boehringer Mannheim, Mannheim, Germany) and stored at -20°C. The presence of the 677C→T polymorphism in MTHFR (SEQ ID NO: 51) was determined by PCR followed by restriction digestion with *HinfI*, as



described (Frosst et al., *Nat. Genet.* 10:111-113, 1995).

### Statistics

Computer-assisted statistical analyses were carried out using SAS for Windows (Version 6.12). Standard summary statistics, analysis of variance, t-tests, calculation of odds ratios with associated confidence limits, and logistic regression models were used where appropriate. Statistical significance was interpreted as p-values of  $p < 0.05$ .

### EXAMPLE II: CLONING OF THE HUMAN METHIONINE SYNTHASE REDUCTASE cDNA

More than 20 overlapping sequences homologous to the FAD and NADPH-binding domains of cytochrome P450 reductase were identified in an initial survey of the NCBI dbEST database using TblastN. We sequenced clones 550341 (accession #AA085543), 704947 (accession #AA279726) and 31776 (accession #R17835) to confirm the sequence of this part of the cDNA. Reprobing the NCBI databases with this sequence yielded a *C. elegans* sequence (accession #Z35595) containing binding sites for FMN, FAD and NADPH. We then used the *C. elegans* sequence to reprobe the dbEST database using TblastN and identified a human sequence (accession #AA192690, clone 628497) containing a putative FMN binding site similar to the one encoded by Z35595. We designed a sense primer based on the FMN binding region of AA192690 and antisense primers corresponding to the FAD/NADPH binding regions of the methionine synthase reductase candidate and amplified a sequence by RT-PCR using human fibroblasts

as the source of RNA. Fig. 2 shows the overlapping clones and PCR fragments used to clone and sequence human methionine synthase reductase. The EST clones are shown as rectangles, the subsequences that were available from the dbEST database are shown as hatched boxes, and the PCR fragments are

5 represented as lines. The oligonucleotide names are indicated below the arrows in Fig. 2 and are described in Table 1 below. The primer in parentheses designates a mispriming outcome that generated valid internal sequence. The letter "V" in black boxes indicates primers annealing to the vector of the cDNA library used as a template for PCR. The presence of a triangle above a segment indicates that it  
10 contained a deletion of 154 bp (open triangle) or 26 bp (black triangle), likely caused by alternative splicing.

Table 1. Oligonucleotides used for cDNA cloning,  
mapping, and mutation detection.

15	<b>Primers</b>	<b>Sequence</b>	<b>Location</b>
	Z116 (SEQ ID NO: 3)	5'-CTCCTGCTCGAACATCTTCCTAAA	1318 - 1341
	Z117 (SEQ ID NO: 4)	5'-AATAGATAATCCCTATCCTTATGCC	1766 - 1742
	AD150 (SEQ ID NO: 5)	5'-CCCTGGCTCCTAAGATATCCATC	1544 - 1566
	AD151 (SEQ ID NO: 6)	5'-CGAACAACAAATTCTTTCCACTTACC	1573 - 1598
20	AB191 (SEQ ID NO: 7)	5'-CAAGGTTGGTGGAAGTCGCGTTG	-79 - -57
	AA468 (SEQ ID NO: 8)	5'-ATGCCTTGAAGTGATGAGGAGGTTT	-13 - 12
	AB586 (SEQ ID NO: 9)	5'-TTCCTACAACATAGAGAGAACTC	1663 - 1686
	AB588 (SEQ ID NO: 10)	5'-TTGCACAAGGGCATCATGTACATC	1998 - 1975
	Z593 (SEQ ID NO: 11)	5'-AAACCTCCTCATCACTTCAAGGCAT	12 - -13
25	Z594 (SEQ ID NO: 12)	5'-CTTGCACACGAATATGGTCTGGG	1370 - 1348
	Z596 (SEQ ID NO: 13)	5'-TGGCATCACCTGCATCCTTGAGG	506 - 528
	Z597 (SEQ ID NO: 14)	5'-GATGTACCTGTAAATATTCTGGGGG	760 - 736
	1103A (SEQ ID NO: 15)	5'-AATCCACGGCTCAACCACAAGTTC	429 - 406



The sequence of the PCR products confirmed that our cDNA contained the putative FMN, FAD and NADPH binding sites. The 5' end of the sequence was obtained by PCR using a cDNA library as template, with antisense primers specific for the cDNA and a sense primer that anneals to the vector used to construct the library. The sequences generated by PCR were taken as error-free by comparison of the sequence of at least two, and usually three, independent PCR reactions.

The coding sequence of human methionine synthase reductase contains 2094 bp (SEQ ID NO: 1 and SEQ ID NO: 41) encoding a polypeptide of 698 amino acids (SEQ ID NO: 2 and SEQ ID NO: 42) in length. Fig. 3 shows the cDNA sequence (SEQ ID NO: 24) and deduced amino acid sequence of human methionine synthase reductase. The nucleotide residues are numbered on the left margin, the amino acids residues are numbered on the right margin, and the stop codon is indicated by three stars. The sequence has been deposited in the GenBank database, accession #AF025794.

The predicted MW of human methionine synthase reductase is 77,700. It shares 38% sequence identity (49% similarity) with human cytochrome P450 reductase (accession #A60557) and 43% identity (53% similarity) with the *C. elegans* putative methionine synthase reductase (accession #Z35595). Fig. 4 shows amino acid sequence comparisons among human methionine synthase reductase (HsMTRR), *C. elegans* putative methionine synthase reductase (CeMTRR) and human cytochrome P450 reductase (HsCPR). The amino acids residues are numbered on the right margin, and conserved residues are shown by stars under the sequence. Alignments of similar amino acids are dotted (A,G,S,T,;

D,E,N,Q; V,L,I,M; K,R; and F,W,Y), and regions proposed to be involved in binding of FMN, FAD or NADPH are shown above the sequences.

The first in-frame methionine residue is a candidate for the initiation codon. It is perfectly aligned with the first methionine of the *C. elegans* sequence, and the presence of a G at positions -3 and -6 places the sequence in good context for initiation of translation (Kozak, *J. Biol. Chem.* 266:19867-19870, 1991). A polyadenylation signal is present at positions 3135-3140. The poly(A) tail is added after position 3165, although we observed some clones with polyadenylation after residue 3157.

RT-PCR involving various pairs of primers allowed us to detect alternatively processed methionine synthase reductase mRNA, including one form with a deletion of 154 bp (nucleotides 129-282) and another lacking a 26 bp segment (-52 to -27), accounting for less than 20% and 40% of the mRNA, respectively.

### EXAMPLE III: EXPRESSION OF HUMAN METHIONINE REDUCTASE mRNA

A PCR product generated with primers 1902C (SEQ ID NO: 19) and 1812B (SEQ ID NO: 18) was subcloned and used to probe a Northern blot prepared from several human tissues.

Figs. 5A and 5B show a Northern blot analysis of methionine synthase reductase expression in human tissues, with the positions of the molecular size (kb) markers indicated at the left. The 1.8 kb probe hybridized to one predominant RNA species of 3.6 kb. Methionine synthase reductase appears to be expressed to

some degree in all tissues tested and is particularly abundant in skeletal muscle. In addition to the 3.6 kb band, a 3.1 kb band and a faint 6 kb band were detected in brain mRNA.

#### EXAMPLE IV: CHROMOSOMAL MAPPING OF THE HUMAN

##### 5 METHIONINE SYNTHASE REDUCTASE GENE

The methionine synthase reductase gene was localized to human chromosome 5, since the gene-specific primer pair amplified a PCR product of the expected size only from the GM10114 hybrid, which contains chromosome 5 as its only human material. Moreover, the DNA sequence we determined for the

10 methionine synthase reductase gene matched markers AA002A03 and STSG444, which were also mapped by the NCBI consortium to chromosome 5 between markers D5S406-D5S478 and D5S406-D5S635, respectively (Hudson, T.J. et al., *Science* 270:1945-1954, 1995). To determine the cytogenetic position of the gene on chromosome 5, we mapped a genomic PAC clone encompassing the gene using

15 fluorescence *in situ* hybridization (FISH). Fig. 6 shows a summary of the FISH mapping of the methionine synthase reductase gene to human chromosome 5p15.2-p15.3. Each dot represents a signal detected on human chromosome 5. The hybridization efficiency was 100%, and, among 100 mitotic figures examined, each result indicated that the gene was located on chromosome 5p15.2-p15.3. We

20 propose MTRR as the gene name for methionine synthase reductase, since the methionine synthase gene has been named MTR.

#### EXAMPLE V: MUTATIONS OF THE METHIONINE SYNTHASE

## REDUCTASE GENE IN PATIENTS OF THE *cblE* COMPLEMENTATION GROUP

To confirm the identity of the candidate cDNA as methionine synthase reductase, patient cell lines from the *cblE* complementation group were analyzed by RT-PCR-dependent heteroduplex analysis using nine RT-PCR reactions that yielded overlapping products, in order to cover the length of the candidate cDNA sequence. Patient samples were mixed with RT-PCR product from normal cells to ensure the availability of wild-type DNA, in order to enable the detection of heteroduplexes in samples in which the mutation might be homozygous. For samples yielding heteroduplexes, the analysis was repeated without prior mixing with wild-type DNA, in order to determine whether the relevant changes were heterozygous. Three cell lines showed typical heteroduplex patterns, one of them observed in overlapping RT-PCR fragments (Fig. 7A and 7B).

Figs. 7A and 7B show a mutation analysis of the methionine synthase reductase gene in *cblE* patient cell lines. Fig. 7A shows the PCR products obtained with primers Z116 (SEQ ID NO: 3) and Z117 (SEQ ID NO: 4) from RT reactions with control sample (WT) and two *cblE* cell lines, WG1146 and WG1836. The bands above the 449 bp amplification product result from heteroduplexes formed between DNA strands bearing different allelic sequences. The pattern observed for cell line WG1146 was also seen with cell line WG788 (the sibling of WG1146). Fig. 7B shows RT-PCR products amplified with primers AB586 (SEQ ID NO: 9) and AB588 (SEQ ID NO: 10) from a control sample and cell line WG1836. Heteroduplexes are observed above the 336 bp band for cell line WG1836.

The heteroduplex-containing samples were subcloned and sequenced and two mutations were identified. A heterozygous mutation present in fibroblast line WG788 is a 4 bp deletion, 1675del4, resulting in a frameshift that creates a nearby stop codon. The same mutation was observed in cell line WG1146 from the brother of patient WG788. Direct sequencing of the PCR product using primer AD150 showed overlapping sequences starting at position 1675, consistent with the heterozygous presence of the 4 bp deletion.

The second heterozygous mutation, detected in cell line WG1836, is an in-frame deletion of 3 bp, 1726delTTG. It results in the loss of a highly conserved leucine at position 576 of the amino acid sequence.

Fig. 7C shows a sequence comparison among proteins of the FNR family in a part of the NADPH binding region in the vicinity of the leucine residue that is deleted in a *cbIE* patient (denoted by a triangle; MTRR is methionine synthase reductase; CPR is cytochrome P450 reductase; NOS is nitric oxide synthase; SR is sulfite reductase; and FNR is NADPH-ferredoxin(flavodoxin) reductase).

Primer AD151 (SEQ ID NO: 6) was used for direct sequencing of the WG1836 PCR product. In this case, the deletion of nucleotides 1726-1728 was clearly visible. There was only a very faint background contributed by the normal sequence, suggesting that a second, unidentified mutation in this cell line was associated with a very low level of steady-state mRNA.

#### EXAMPLE VI: HUMAN METHIONINE SYNTHASE REDUCTASE POLYMORPHISMS



We have identified two polymorphisms in methionine synthase reductase cDNAs. The first is a G/A polymorphism at nucleotide position 66, using the "A" of the initiator methionine as nucleotide position number 1 (see Fig. 3), which results in either an isoleucine or a methionine, respectively, at amino acid 22. The second polymorphism is a G/A polymorphism at nucleotide position 110, which results in either a tyrosine or a cysteine, respectively, at amino acid position 37. It is likely that additional methionine synthase reductase polymorphisms will be found, some of which will be associated with increased or decreased risks of disease.

#### 10 EXAMPLE VII: A COMMON POLYMORPHISM IN METHIONINE SYNTHASE REDUCTASE AS A RISK FACTOR FOR SPINA BIFIDA

During screening for methionine synthase reductase (MTRR) mutations in patients with homocystinuria, we identified an A/G polymorphism at bp 66, which yields an isoleucine (22I) or a methionine (22M), respectively, at amino acid position 22 (Figure 8A). Since the presence of the methionine polymorphism at this position did not create or obliterate a naturally-occurring restriction site, a PCR-dependent diagnostic test was established that makes use of a modified sense primer to create a NdeI site in the isoleucine allele during the amplification reaction. The PCR product of 66 bp remains uncut in the presence of the methionine allele, but is digested into fragments of 44 and 22 bp in the presence of the isoleucine allele (Figure 8B). The cDNA sequence reported in Leclerc, et al., *Proc. Natl. Acad. Sci. USA*, **95**:3059-3064, 1998, contained the methionine codon.

The NdeI assay was used to assess allele frequencies in controls. The

22I/22M polymorphism was extremely common in our control adult population (mothers of control children, n=89). Forty-nine percent were heterozygous while 26% were homozygous for the methionine allele (Table 2). The allele frequency was 0.51 for the methionine variant. Similar frequencies were observed for control children. The controls in this study were white Caucasian individuals with French, British, and mixed European ancestry. Since the allele frequency is virtually identical for the two variants, the designation of a "wild type" allele could not be ascertained based on frequency. However, this gene has significant homology with related FMN-binding proteins from other organisms, including the putative methionine synthase reductase from *C. elegans*, as well as sulfite reductases, nitric oxide synthases, cytochrome P450 reductases, and flavodoxins. The equivalent codon in these genes is isoleucine, leucine, or valine in 123 out of 130 entries in GenBank. None of the entries contained a methionine codon. Consequently, the ancestral human MTRR sequence is likely to contain the isoleucine codon (22I), with a subsequent mutation to methionine (22M).

In this study, 34% (19/56) of case (spina bifida) children and 36% (21/58) of case mothers were homozygous for the 22M polymorphism in MTRR, compared to 30% (29/97) of control children and 26% (23/89) of control mothers (Table 2). An increased risk for being a case (odds ratio (OR) 1.7, 95% confidence interval (CI) 0.67-4.6) or a case mother (O.R. 2.0, 95% CI 0.77-5.2) was observed when the homozygous mutant (M/M) genotype was present, but this increase was not statistically significant. Mother-child genotype pairs were also assessed for neural tube defect (NTD) risk to determine if the combination of mutant maternal and mutant child genotypes conferred a greater risk than either genotype alone; an

increased risk was not observed. Homocysteine levels were not increased in individuals who were homozygous mutant for MTRR (Table 3).

*Synergistic interaction between MTRR genotype and cobalamin level influences the risk of NTD*

- 5 Case children had serum cobalamin levels (pmol/L) of  $487 \pm 250$  (n=55), whereas control children had serum cobalamin levels of  $535 \pm 339$  (n=95); case mothers had serum cobalamin levels of  $298 \pm 186$  (n=59), whereas control mothers had serum cobalamin levels of  $350 \pm 135$  (n=88; p=0.05). We therefore asked whether the mutant MTRR genotype may have a greater impact on NTD
- 10 risk when cobalamin levels are low. Table 4 shows the results of multiple logistic regression analysis, adjusted for age, to test this hypothesis. Having a cobalamin level in the lowest quartile of the control distribution was associated with a nonsignificant two-fold increase in risk for the case mothers (O.R. = 2.1; 95% CI = 0.86-5.2). There was no increase in risk for low cobalamin in the children.
- 15 However, the combination of homozygous mutant genotype and low cobalamin was associated with a significant 5-fold increase in risk for the mothers, compared to those without the M/M genotype and with cobalamin levels in the other 3 quartiles (O.R. = 4.8, 95% CI = 1.5 - 15.8). The risk for the children with this combination was also increased but statistical significance was not observed (O.R. = 2.5, 95% CI = 0.63 - 9.7). There was no increased risk for the mutant genotype combined with low folate. Because the MTRR genotype alone was associated with less risk, we speculate that genotype and cobalamin levels work in unison to produce increased risk for spina bifida in the case mothers and case children.
- 20

*Synergistic interaction between MTRR and MTHFR genotypes influences the risk of NTD*

The 677C→T polymorphism (SEQ ID NO: 51) in the  
5 methylenetetrahydrofolate reductase (MTHFR) gene converts an alanine to a  
valine residue in the enzyme (Frosst et al., *Nat. Genet.* 10:111-113, 1995).  
MTHFR catalyzes the synthesis of 5-methyltetrahydrofolate, the primary  
circulatory form of folate and the methyl donor in the remethylation of  
homocysteine to methionine by methionine synthase. Several studies have  
10 demonstrated an increased frequency of the homozygous mutant (V/V) MTHFR  
genotype in children with NTDs and in their mothers (van der Put et al., *Lancet*  
346:1070-1071, 1995; Whitehead et al., *Quart. J. Med.* 88:763-766, 1995; Ou et  
al., *Am. J. Med. Genet.* 63:610-614, 1996).

Table 5 shows the interaction between the MTRR genotype and the  
15 MTHFR genotype in NTD risk, as determined by multiple logistic regression  
analysis, adjusted for age. Using a genotype of either homozygous wild type or  
heterozygous for MTRR and homozygous wild type for MTHFR as the reference,  
a risk nearly five times as great is conferred to case children (O.R. = 4.9, 95%  
CI=1.1-21.8) and to case mothers (O.R.= 5.0 , 95% CI 0.8 - 31.3) when they are  
20 homozygous for both mutations. The risk for the combination of mutant genotypes  
is clearly higher than either mutant genotype alone, in both the cases and in their  
mothers.

Table 2. Frequency of MTRR genotypes in children with spina bifida (cases) and in case mothers.			
	I/I	I/M	M/M
Cases	9/56 (16%)	28/56 (50%)	19/56 (34%)
Controls	24/97 (25%)	44/97 (45%)	29/97 (30%)
Case mothers	10/58 (17%)	27/58 (47%)	21/58 (36%)
Control mothers	22/89 (25%)	44/89 (49%)	23/89 (26%)
O.R. for children, M/M vs. I/I = 1.7 (95% C.I. 0.67-4.6)			
O.R. for mothers, M/M vs. I/I = 2.0 (95% C.I. 0.77-5.2)			

Table 3. Homocysteine levels stratified by MTRR genotype. (tHcy (μmol/L))			
	I/I	I/M	M/M
	n	n	n
Children	7.7 ± 2.8 33	8.2 ± 3.3 72	8.2 ± 3.1 48
Mothers	9.7 ± 2.8 32	10.3 ± 4.7 71	9.4 ± 3.1 43

Table 4. Logistic regression analysis for NTD risk in children and mothers. Odds ratio > (95% C.I.)			
MTRR Genotype	Cobalamin level	Children	Mothers
I/I or I/M	normal	1.0 (ref.)	1.0 (ref.)
I/I or I/M	low	0.92 (0.37-2.3)	2.1 (0.86-5.2)

M/M	normal	1.1 (0.46-2.5)	1.5 (0.56-4.1)
M/M	low	2.5 (0.63-9.7)	4.8 (1.5-15.8)
Odds ratios are adjusted by age of children and mothers respectively. Low cobalamin refers to the lowest quartile of the control distribution; normal refers to the other 3 quartiles.			

5

Table 5. Logistic regression analysis for NTD risk in children and mothers. Odds ratio > (95% C.I.)			
MTRR Genotype	MTHFR Genotype	Children	Mothers
I/I or I/M	A/A	1.0 (ref.)	1.0 (ref.)
I/I or I/M	V/V	0.82 (0.18-3.7)	2.4 (0.69-8.3)
M/M	A/A	1.2 (0.34-4.5)	1.9 (0.61-5.7)
M/M	V/V	4.9 (1.1-21.8)	5.0 (0.80-31.3)
Odds ratios are adjusted by age of children and mothers respectively.			

10

EXAMPLE VIII: HUMAN METHIONINE SYNTHASE REDUCTASE  
MUTATIONS AND POLYMORPHISMS IN DISEASE

Alterations in metabolism of folates, homocysteine, methionine, vitamin B12, and S-adenosylmethionine are associated with diseases such as megaloblastic anemia and conditions such as hyperhomocysteinemia. In turn, hyperhomocysteinemia may be associated with a higher than normal risk for cardiovascular disease and neural tube defects. In addition, decreased folate levels may be predictive of a lower than normal risk for cancer.

DNA samples from patients having a disease or developmental defect, such as those mentioned above, are analyzed for mutations within the methionine synthase reductase coding region and/or transcriptional control regions, and serum folate, red blood cell folate, plasma homocysteine, and serum cobalamin levels are measured. Patient samples are compared to control samples.

The cloning of the methionine synthase reductase gene makes possible the determination of whether discrete mutations and polymorphisms in methionine synthase reductase nucleic acid confer an increased risk for, or in contrast, protection against, diseases and conditions such as cardiovascular disease, cancer, and neural tube defects, (those of skill in the art will understand that polymorphisms and mutations may either increase or decrease the relative risk of any given disease or developmental defect). This collection of data in turn makes possible the development of diagnostic assays that predict whether a subject has a higher than normal risk of developing a disease or of having offspring with developmental defects. An understanding of disease-enhancing or -protective mutations allows the development of therapeutics that appropriately modulate

methionine synthase reductase activity.

EXAMPLE IX: ASSOCIATION BETWEEN VARIANTS IN MTHFR AND/OR MTRR WITH DOWN'S SYNDROME

We have identified an association between the identified polymorphism  
5 in methionine synthase reductase (MTRR) (an A → G polymorphism at nucleotide  
position 66), the identified polymorphism in methylenetetrahydrofolate reductase  
(MTHFR) (a C→T polymorphism at nucleotide 677 (SEQ ID NO: 51) that  
converts an alanine to a valine residue (Frosst et al., *supra*)) and Down's  
Syndrome. In the study presented in Table 6, the genotypes of mothers of Down's  
10 Syndrome babies (DS mother) were compared to the genotypes of mothers of  
normal babies. We found that mothers of Down's Syndrome babies had a  
significant 2.49-fold greater likelihood of having a homozygous mutation for the A  
→ G polymorphism at nucleotide position 66. In addition, we found that mothers  
of Down's Syndrome babies had a 2.07 fold greater likelihood of having a  
15 heterozygous mutation or a homozygous mutation in the MTHFR gene. Finally,  
we identified a positive interaction between the MTRR and MTHFR gene  
mutations. Table 6 demonstrates that mothers with Down's Syndrome babies had  
an even greater likelihood of having both the MTRR and MTHFR mutations than  
having either the MTRR or MTHFR mutations alone. Mothers with Down's  
20 Syndrome babies had a 3.71 fold greater likelihood of having both a MTRR and a  
MTHFR mutation than control mothers. This result indicates that the identified  
mutations are useful as genetic markers for detection of Down's Syndrome in a  
fetus or embryo. Alternatively, these mutations can be used to assess the risk of a



particular mother of having a Down's Syndrome baby.

#### EXAMPLE X: INCREASED RISK FOR PREMATURE CORONARY ARTERY DISEASE

We investigated whether an A66G polymorphism in the MTRR gene is associated with altered levels of homocysteine and/or the risk of developing premature coronary artery disease. The findings described below suggest that the methionine synthase reductase homozygous GG genotype is a risk factor for the development of premature coronary artery disease (Relative risk 1.49; 95% CI: 1.10-2.03), by a mechanism independent of the detrimental vascular effects of hyperhomocysteinemia.

Four hundred seventy eight Caucasian individuals undergoing cardiac catheterization procedures at the Carolinas Heart Institute were recruited into the study. All patient volunteers provided blood samples for the isolation of serum, plasma and DNA. Of the 478 consenting participants, 463 had complete MTRR genotype data (96.86%), and 180 of these patients were at risk for premature coronary artery disease (CAD) by having ages < 58 years (38.88%). A total of 124 of these individuals at risk (66.67%) had premature coronary artery disease (CAD) with significant atherosclerosis, defined as  $\geq 50\%$  occlusion of  $\geq 1$  major artery or 20-50% occlusions in each of  $> 2$  major arteries. The remaining 62 individuals (33.33%) were free of significant occlusions ( $< 50\%$  occlusion in  $\leq 1$  major artery), and therefore were defined as controls. Among the individuals with premature CAD 21/124 (16.94%) were female and 103/124 (83.06%) were male. Of the 116/180 age-eligible study participants reporting ethnicity (64.44%), the majority

were of British or German descent. A summary of the characteristics of the population of individuals <58 years of age is presented in Table 7.

Arterial blood was collected in ACD (acid-citrate-dextrose) and serum vacutainer tubes (Beckton-Dickinson, Franklin Lanes, NJ) and immediately placed on ice (for <2 hours) prior to centrifugation at 3000 rpm for 20 minutes. Plasma and serum were removed and stored in screw cap cryovials at 70°C. Plasma homocysteine, serum folate, and vitamin B<sub>12</sub> concentrations were measured at the Vascular Disease Intervention and Research Laboratory at the Oklahoma University Health Science Center.

The region surrounding the MTRR 66A→G polymorphism was amplified by the polymerase chain reaction using primers A:5'-CAGGCAAAGGCCATCGCAGAAGACAT-3' (SEQ ID NO: 61) and B:5'-CACTTCCCAACCAAAATTCTTCAAAAG-3' (SEQ ID NO: 62). The amplifications were performed in a 50µl volume, containing 200 ng genomic DNA, 10mM TRIS, pH 8.8, 50 mM KCl, 1 mM each dNTP, 1 µM each primer and 2.5 U Taq polymerase (Perkin-Elmer, Norwak, CT). PCR cycling conditions in a GeneAmp 2400 thermal cycler (Perkin-Elmer, Norwak, CT) were: 94°C for 5 minutes, followed by 30 cycles of 94°C for 0.5 minutes, 55°C for 0.5 minutes, 72°C for 0.5 minutes, and a final extension of 72°C for 5 minutes. Primer A, containing a mismatch from the MTRR sequence (underlined) creates a *Nde*I site in the amplified DNA from alleles containing the 66A→G polymorphism, digesting the 150 bp amplimer into 123 and 27 bp fragments. The fragments were separated on a 2% Nusieve/1% agarose gel containing 0.6µg/ml ethidium bromide.

Statistical analyses were performed using Stata Statistical Software

(College Station, TX). Contingency table analysis with 3-levels of genotype were used for comparison of disease or genotype frequencies between groups, with sided p-values from Pearson chi-square tests or from Fisher's Exact Test where expected cell frequencies were  $\leq 5$ , trend tests from Cuzick's non-parametric test (Cuzick J.A, Statistics in Medicine (1985) 4:87-90) and non-parametric adjustments of relative risks with the Mantel-Haenszel procedure. Kruskal-Wallace tests or analysis of variance of lag transformed measurements was used to test for differences in plasma tHcy, and serum folate and vitamin B<sub>12</sub> levels between the three MTRR genotype levels, respectively. Logistic regression was used to model risk of premature CAD adjusted for other covariates. Descriptive statistics including means and standard deviations or counts and percentages were calculated. A p-value of less than 0.05 was considered statistically significant.

We found that MTRR genotype analysis from 58 healthy, unselected Caucasian individuals from North Carolina revealed a genotype distribution of 22.4% AA, 50% AG, and 27.6% GG, indicating the high frequency of the 66A→G polymorphism in the local population.

We then determined if the MTRR genotype was associated with premature CAD in our population of patients undergoing cardiac catheterization procedures. A 3 X 2 contingency table analysis displayed an association between premature CAD and both male sex and MTRR genotype ( $p < 0.0001$ ) (Table 8). Among both males and females, individuals with the homozygous GG genotype were at greatest risk of developing premature CAD. Relative risks (RR) of premature CAD, with Mantel-Haenszel adjustment for sex, were RR = 1.49 (95% CI: 1.10, 2.03) for GG versus AA and RR = 1.21 (95% CI: 0.88, 1.65) for AG

versus AA. Cuzick's non-parametric test for trend in premature CAD risk across the ordered genotype groups yielded a p-value of  $p = 0.03$ .

A stratified analysis detected no appreciable modification of the association between MTRR GG genotype and premature CAD by MTHFR TT genotype, with a Mantel-Haenszel adjustment relative risk for premature CAD for the MTRR GG versus MTRR AA genotypes across MTHFR strata of 1.47 (95% CI: 1.04-2.06) compared to the crude relative risk of premature CAD of 1.38 for the MTRR GG versus MTRR AA genotypes.

As summarized in Table 9, no substantial differences in the mean fasting plasm tHcy, serum folate, or vitamin B<sub>12</sub> concentrations between the three MTRR genotype levels were detected. Non-parametric Kruskal-Wallis tests for difference in the distributions of continuous covariates yielded p-values of 0.65 for tHcy, 0.18 for folate, and 0.69 for vitamin B<sub>12</sub>. ANOVA models predicting log-transformed continuous variables with adjustment for sex yielded p-values for MTRR level of 0.62 for tHcy, 0.25 for folate, and 0.79 for vitamin B<sub>12</sub>.

We next examined the influence of vitamin B<sub>12</sub> status on the association between MTRR genotype and premature CAD as well as with homocysteine level. The proportion of individuals with premature CAD within the three MTRR genotype groups did not differ among those with vitamin B<sub>12</sub> levels above and below the median value of 300 pg/mL (AA-58.8% versus 55.6%, n=35; AG-63.8% versus 67.5%, n=87; GG-77.3% versus 80.8%, n=48). The overall p-value for premature CAD by vitamin B<sub>12</sub> levels above and below the median was 0.91, and adjustment for sex and MTRR level via logistic regression yielded a p-value for vitamin B<sub>12</sub> levels above and below the median of 0.79.

When combining case and control individuals, those with B<sub>12</sub> values below the median were found to have higher tHcy concentrations (p=0.003). Individuals with B<sub>12</sub> values below the median had higher tHcy concentrations within each stratum of MTRR genotype. The differences in μmol/L and p-values from Wilcoxon on rank sum tests for AA, AG and GG genotypes were 1.3 (p=0.049), 1.5 (p=0.031), and 1.6 (p=0.35), respectively.

The MTRR GG genotype was significantly associated with premature onset coronary artery disease in the study population. This association of genotype with disease was not modulated by vitamin B<sub>12</sub> status or MTHFR genotype.

- Without limiting the biochemical mechanism of the invention, we propose that the mechanism by which possession of the GG genotype predisposes a subject to CAD does not appear to be related to the effects of hyperhomocysteinemia, as there was no difference in tHcy concentrations between the MTRR genotype levels. An inverse relationship between vitamin B<sub>12</sub> concentration and tHcy levels was detected within the MTRR genotype groups, supporting previous reports of an inverse relationship between homocysteine and vitamin B<sub>12</sub> levels (Verhoef et al. *Am. J. Epidemiol* (1996) 143:845-859; Folsom et al., *Circulation* (1998) 98:204-210).

- These results indicate that the identified mutations are useful as genetic markers for detection of premature cardiovascular disease in a fetus or embryo. Alternatively, these mutations can be used to assess the risk of a particular mother of having a baby that might, in the future, develop premature cardiovascular disease.

Table 6: Association between variants in MTHFR or MTRR or both with Down's Syndrome (DS)

Interaction between MTHFR and MTRR gene mutations

MTHFR	MTRR	control	DS mother	Odds ratio (95% CI)
-	-	55	29	1 (reference)
+	-	59	65	2.07 (1.17-3.66)
-	+	15	20	2.49 (1.12-5.52)
+	+	19	38	3.71 (1.84-7.51)
Total		148	152	

10 MTHFR- = Homozygous normal

MTHFR+ = Heterozygous mutation and homozygous mutation combined

MTRR- = Homozygous normal and heterozygous mutation combined

MTRR+ = Homozygous mutation

15 Table 7. Comparison of characteristics among cases with coronary arteriosclerosis versus control subjects.

Individuals <58 years of age

(n=180)

	Patients	Controls	
Sex - Male	99/119 (83.2%)	32/61 (52.5%)	P<0.001
-Female	20/119 (16.8%)	29/61 (47.5%)	
Hypercholesterolemia	84/114 (73.7%)	33/59 (55.9%)	P=0.03

Hypertension	69/117 (59.0%)	33/60 (55.0%)	P=0.63
Diabetes	26/118 (22.0%)	8/61 (13.1%)	P=0.17
Current smoker	36/119 (30.3%)	15/61 (24.6%)	P=0.49

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Table 8: Percentage of males and females <58 years of age with CAD by MTRR genotype.

MTRR Genotype	Males		Females	
	Cases	Controls	Cases	Controls
AA	64.5%	35.5%	16.7%	83.3%
	(n=20)	(n=11)	(n=1)	(n=5)
AG	73.4%	26.6%	39.3%	60.7%
	(n=47)	(n=17)	(n=11)	(n=17)
GG	88.9%	11.1%	53.3%	46.7%
	(n=32)	(n=4)	(n=8)	(n=7)

Cuzick's non-parametric test for trend in premature CAD risk across the ordered genotype groups yielded a p-value of p=0.03. RR of premature CAD=1.49 (95% CI:1.10, 2.03) for GG versus AA.



Table 9: Distribution of tHcy, folate and vitamin B<sub>12</sub> concentrations by sex and MTRR genotype.

	MTRR AA		MTRR AG		MTRR GG	
	Male	Female	Male	Female	Male	Female
Plasma tHcy ( $\mu\text{mol/L}$ )	11.0 $\pm$ 2.3	10.0 $\pm$ 3.0	12.2 $\pm$ 4.6	9.5 $\pm$ 3.1	11.1 $\pm$ 4.4	9.8 $\pm$ 2.8
	(n=30)	(n=6)	(n=63)	(n=28)	(n=35)	(n=15)
Serum folate (ng/mL)	16.3 $\pm$ 8.4	13.3 $\pm$ 7.5	13.4 $\pm$ 7.8	13.7 $\pm$ 9.5	14.0 $\pm$ 7.1	14.1 $\pm$ 5.2
	(n=31)	(n=6)	(n=64)	(n=28)	(n=35)	(n=15)
Serum vitamin B <sub>12</sub> (pg/mL)	338.1 $\pm$ 153.1	260.4 $\pm$ 66.4	350.8 $\pm$ 192.1	334.4 $\pm$ 176.6	320.4 $\pm$ 132.8	289.4 $\pm$ 88.7
	(n=30)	(n=5)	(n=59)	(n=28)	(n=35)	(n=13)

p-values of 0.65 for tHcy, 0.18 for folate, and 0.69 for vitamin B<sub>12</sub> (Kruskal-Wallis tests);

p-values for 0.62 for tHcy, 0.25 for folate, and 0.79 for vitamin B<sub>12</sub> (ANOVA models predicting log-transformed continuous variables with adjustment for sex)

What is claimed is: